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Turtles as a Possible Reservoir of Nontyphoidal *Salmonella* in Shanghai, China

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Abstract

Terrapins and turtles are known to transmit *Salmonella* to humans. However, little was known about the occurrence of this pathogen in soft-shelled terrapin that is a popular delicacy in Chinese and other East Asian cuisines. We isolated and characterized 82 (24.4%) isolates of *Salmonella* from 336 fecal samples of soft-shelled terrapins (51 of 172; 29.7%) and pet turtles (31 of 164; 18.9%) in Shanghai. *Salmonella* Thompson was the most common serotype (17.1%) among others. Many isolates (84.1%) were resistant to multiple antimicrobials (≥3). Molecular analysis of *Salmonella* Thompson and *Salmonella* Typhimurium using pulsed-field gel electrophoresis unveiled a close genetic relationship between several human and terrapin isolates. Our results highlight the risk associated with the handling and consumption of turtles and their role in the spread of *Salmonella* in the human salmonellosis.

Introduction

Salmonella is a zoonotic pathogen capable of causing foodborne disease, which is a significant public health concern. It is estimated that *Salmonella* causes annually 93.8 million illnesses and 155,000 deaths worldwide and that 80.3 million cases are linked to ingestion of *Salmonella*-contaminated food (Majowicz *et al.*, 2010). Food commodities frequently associated with large-scale *Salmonella* outbreaks include poultry, shell egg, pork, beef, fruit, and fresh produce (Brands *et al.*, 2005; Kenney *et al.*, 2006; Zhao *et al.*, 2008; Yan *et al.*, 2010). Recently, spices and other types of food have emerged as novel

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transmission vehicles for *Salmonella* in several outbreaks, highlighting the urgent need of screening more food commodities for potential risks of salmonellosis (Carrasco, 2012; Zweifel *et al.*, 2012).

As a carrier of *Salmonella*, turtles are a well-recognized source of human salmonellosis. Humans may acquire *Salmonella* by direct or indirect contact with turtles (Bertrand *et al.*, 2008; Harris *et al.*, 2010). Pet turtles have been responsible for several outbreaks of human salmonellosis in the United States (Harris *et al.*, 2010). In China, soft-shelled terrapin (*Pelodiscus sinensis*) is cultivated for food; the meat, skin, and internal organs are used for cooking turtle soup, a popular delicacy in Chinese and other East Asian cuisines. According to a survey of 684 Chinese turtle farms, more than 91 million turtles of this species are produced each year for human consumption (Shi *et al.*, 2008). However, there is no information on pet turtles available in China. Given the large number of soft-shelled terrapins sold for human consumption in China and the known risk, a study was conducted to examine the prevalence of *Salmonella* in soft-shelled terrapins and pet turtles in Shanghai, China. *Salmonella* isolates were examined for resistance to antimicrobials and subtyped of the two most common serotypes in turtles and humans using pulsed-field gel electrophoresis (PFGE) to assess the genetic relatedness between human and turtle isolates, which could provide insightful data for developing appropriate intervention and control strategies.

Materials and Methods

Bacterial isolates

Between March 2008 and December 2011, a total of 336 fecal samples were collected from soft-shelled terrapins (n = 172) and pet turtles (n = 164) in supermarkets and farmer's markets in Shanghai on a biweekly basis using cotton swabs. *Salmonella* was isolated following standardized procedure recommended by the World Health Organization (WHO, 2010). Typical *Salmonella* isolates were further identified with API identification kits (BioMérieux, Marcy l'Étoile, France).

In addition, from 2006 to 2011, 34 clinical isolates from humans (14 *Salmonella* Thompson and 20 *Salmonella* Typhimurium) collected from the Shanghai Center for Disease Control and Prevention were also included in the study. The detailed information, including *Salmonella* isolation, identification, and serotyping, was published previously (Zhang *et al.*, 2015).

Serotyping

All isolates were serotyped in the Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China. O and H antigens were characterized using slide agglutination with hyperimmune sera (S&A Reagents Lab, Bangkok, Thailand), and the serotype was assigned following the manufacturer's instructions.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of antimicrobials were determined by the agar dilution method and interpreted according to the Clinical and Laboratory Standards

Institute's (CLSI) guidelines (CLSI, 2003). The following antimicrobials were tested: ampicillin (AMP), amoxicillin–clavulanic acid (AMC), ceftriaxone (CRO), chloramphenicol (CHL), nalidixic acid (NAL), ciprofloxacin (CIP), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), tetracycline (TET), sulfisoxazole (SUL), and trimethoprim–sulfamethoxazole (SXT). *Escherichia coli* ATCC25922 and ATCC35218 were used as quality control organisms in the MIC determinations. Breakpoints for most antimicrobials were used according to the interpretive standards by the CLSI (2010). The breakpoint for streptomycin was chosen from a previous study (Chen *et al.*, 2004).

Pulsed-field gel electrophoresis

PFGE analysis was performed according to the protocol developed by the Centers for Disease Control and Prevention (Ribot *et al.*, 2006). Briefly, agarose-embedded DNA was digested with 50 U of *Xba*I (TaKaRa, Dalian, China) for 1.5–2 h in a water bath at 37°C. Restriction fragments were separated by electrophoresis in 0.5 × TBE buffer at 14°C for 18 h using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times of 2.16–63.8 s. *Salmonella enterica* serotype Braenderup H9812 was used as the molecular weight size standard. The gels were stained with ethidium bromide, and DNA bands were visualized with UV transillumination (Bio-Rad). PFGE results were analyzed using BioNumerics software (Applied Maths, Kortrijk, Belgium).

Statistical analysis

The chi-square or Fisher's exact test was used for data analysis using SAS 9.2 (SAS Institute, Cary, NC). A p-value <0.05 was considered statistically significant.

Results and Discussion

Salmonella was detected more frequently in soft-shelled terrapins (51 of 172; 29.7%) than in pet turtle samples (31 of 164; 18.9%) ($p < 0.05$; Table 1). Twenty-two serotypes of *Salmonella enterica* were identified. In the top nine serotypes, *Salmonella* Thompson was the most common serotype in both reptiles (15.7% soft-shelled terrapin and 19.4% pet turtles). Seven other serotypes (*Salmonella* serotypes: Hvittingfoss, Typhimurium, Wandsworth, Stanley, Saintpaul, Singapore, and Kedougou) were also found in both reptiles. *Salmonella* Virchow was relatively abundant in soft-shelled terrapins (9.8%) but not isolated in pet turtles, whereas *Salmonella* Stanley was more recovered from pet turtles (12.9%) than from soft-shelled terrapins (2.0%).

The 82 *Salmonella* isolates were all resistant to sulfamethoxazole (100%) and almost all to trimethoprim–sulfamethoxazole (96%). Resistance to tetracycline (70%), ampicillin (63%), kanamycin (62%), and amoxicillin–clavulanic acid (51%) was observed in more than half of the isolates, while resistance to chloramphenicol (43%), streptomycin (34%), nalidixic acid (27%), gentamicin (12%), ciprofloxacin (6.1%), and ceftriaxone (3.7%) was less pronounced. *Salmonella* isolates from pet turtles showed equal or higher resistance rates than those from soft-shelled terrapins to all antimicrobials, except for quinolones (Table 2). 84.1% were resistant to at least three antimicrobial agents tested. Five *Salmonella* isolates resistant to ciprofloxacin were recovered from soft-shelled terrapins, three of which were

Salmonella Thompson. Comparison of the antimicrobial profiles of the 21 *Salmonella* Thompson and *Salmonella* Typhimurium isolates revealed 16 different profiles (Table 3). The majority of these isolates (93% *Salmonella* Thompson and 86% *Salmonella* Typhimurium) were resistant to at least six of the antimicrobials tested.

To identify if turtles are a potential reservoir of *Salmonella* for human salmonellosis, 34 clinical isolates from humans (14 *Salmonella* Thompson and 20 *Salmonella* Typhimurium) from the Shanghai Center for Disease Control and Prevention were compared with 21 turtle isolates (14 *Salmonella* Thompson and 7 *Salmonella* Typhimurium) by PFGE analysis. The 28 *Salmonella* Thompson and 27 *Salmonella* Typhimurium isolates yielded 19 and 16 distinctive patterns, respectively (Fig. 1). At a similarity value of 80%, isolates in both serotypes formed two branches: one branch consisted of a single soft-shell terrapin isolate (*Salmonella* Thompson SH10SF131), while the other branch included the remaining isolates. The two largest clusters in *Salmonella* Thompson were interspersed with human and turtle isolates, although several isolates (such as SH09SF019, SH09SF005, and SH07G039) had a similar PFGE profile (Fig. 1A). In *Salmonella* Typhimurium, three clusters contained a mixture of human and turtle isolates, but only one human isolate (SH10G178) and one isolate from soft-shelled turtle (SH11SF193) had the same PFGE profile (Fig. 1B).

The results showed that *Salmonella enterica* could be recovered from fecal samples of approximately one quarter of retail soft-shelled terrapins and pet turtles tested in Shanghai and that genetic relatedness between isolates recovered from humans and the reptiles suggests a possible public health risk of *Salmonella* infections transmitted through turtles. Four serotypes (*Salmonella* serotypes: Thompson, Hvittingfoss, Typhimurium, and Wandsworth) accounted for 44% of the turtle isolates. *Salmonella* Thompson and *Salmonella* Typhimurium were also among the four most frequently recovered serotypes from human patients in Shanghai (Zhang *et al.*, 2014). Although *Salmonella* Hvittingfoss is a rare serotype globally, it has been implicated in several foodborne outbreaks in the United States and Australia (Oxenford *et al.*, 2005; Falkenstein, 2013). *Salmonella* Wandsworth is also a rare serotype in the world. However in Shanghai, *Salmonella* Wandsworth was the second most common serotype in retail aquaculture products. *Salmonella* Wandsworth was isolated from many different aquaculture products, except shellfish, showing its wide distribution in nature in Shanghai (Zhang *et al.*, 2015). Multidrug resistance of the *Salmonella* isolates, particularly to ciprofloxacin and ceftriaxone, two clinically important antibiotics commonly prescribed for salmonellosis treatment, is alarming. Similar resistance profiles were also observed among human clinical isolates recovered in Shanghai (Zhang *et al.*, 2014). The potential role of soft-shelled terrapins and pet turtles in human salmonellosis in Shanghai was further evidenced by a close genetic relatedness and identical PFGE patterns shared between the animal and clinical isolates. Notably, identical clones of *Salmonella* were recovered from different origins in 1- to 3-year intervals, suggesting the persistence of these pathogens in the local area.

Conclusions

In summary, our findings indicate that turtle used for food or as pets may be an important reservoir for *Salmonella*. Precaution needs to be taken during processing and handling of turtles to reduce risk of human salmonellosis.

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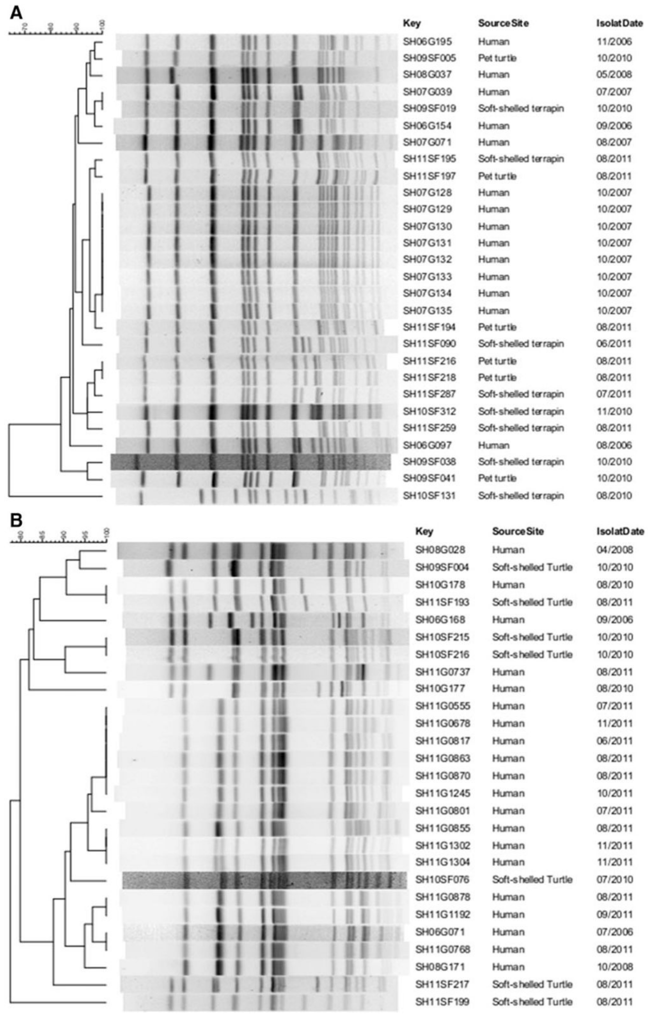

FIG. 1.

Dendrogram of PFGE showing clonal relationships of *Salmonella* Thompson **(A)** and *Salmonella* Typhimurium **(B)** collected from turtles and humans. PFGE, pulsed-field gel electrophoresis.

Table 1

Salmonella enterica Serotypes Isolated from Retail Soft-Shelled Terrapins and Pet Turtles in Shanghai, China

	***Salmonella* isolates, *n* (%)**		
***Salmonella* serotypes**	**Soft-shelled terrapins (*n* = 51)**	**Pet turtles (*n* = 31)**	**Total (*N* = 82)**
Thompson	8 (15.7)	6 (19.4)	14 (17.1)
Hvittingfoss	4 (7.8)	4 (12.9)	8 (9.8)
Typhimurium	4 (7.8)	3 (9.7)	7 (8.5)
Wandsworth	5 (9.8)	2 (6.5)	7 (8.5)
Virchow	5 (9.8)	0 (0)	5 (6.1)
Stanley	1 (2.0)	4 (12.9)	5 (6.1)
Saintpaul	3 (5.9)	2 (6.5)	5 (6.1)
Singapore	3 (5.9)	1 (3.2)	4 (4.9)
Kedougou	3 (5.9)	1 (3.2)	4 (4.9)
Other subtypes	15 (29.4)	8 (25.8)	23 (28.0)

Table 2

Antimicrobial Resistance Among *Salmonella* Isolated from Soft-Shelled Terrapins and Pet Turtles

	Number of antimicrobial-resistant isolates among(%)						
	All *Salmonella*(%)			*Salmonella* Thompson(%)		*Salmonella* Typhimurium(%)	
Antimicrobials	**Combined (N = 82)**	**Terrapin (n = 51)**	**Pet turtle (n = 31)**	**Terrapin (n = 8)**	**Pet turtle (n = 6)**	**Terrapin (n = 4)**	**Pet turtle (n = 3)**
Aminoglycosides							
Gentamicin	12.2	11.8	12.9	12.5	33.3	50	0
Kanamycin	62.2	54.9	74.2	62.5	100	100	66.7
Streptomycin	34.1	29.4	41.9	62.5	66.7	25	66.7
Aminopenicillins							
Ampicillin	63.4	54.9	77.4	75	83.3	100	33.3
β-Lactamase inhibitor							
Amoxicillin–clavulanic acid	51.2	41.2	61.3	75	50	75	33.3
Cephems							
Ceftriaxone	3.7	2.0	6.5	12.5	33.3	0	0
Folate pathway inhibitors							
Trimethoprim–sulfamethoxazole	96.3	94.1	100	100	100	100	100
Sulfamethoxazole	100	100	100	100	100	100	100
Phenicols							
Chloramphenicol	42.7	41.2	45.2	62.5	83.3	100	100
Quinolones							
Ciprofloxacin	6.1	9.8	0	25	0	50	0
Nalidixic acid	26.8	29.4	22.6	12.5	33.3	75	33.3
Tetracyclines							
Tetracycline	69.5	60.8	83.9	87.5	100	50	66.7

Table 3

Antimicrobial Resistance Profiles of *Salmonella* Thompson and *Salmonella* Typhimurium Isolates from Soft-Shelled Terrapins and Pet Turtles

	Number of isolates possessing a specific profile(%)			
	Salmonella Thompson(%)		*Salmonella* Typhimurium(%)	
Resistance profiles	**Terrapin (n = 8)**	**Turtle (n = 6)**	**Terrapin (n = 4)**	**Turtle (n = 3)**
AMP-AUG-CHL-CIP-CRO-SMX-STR-SXT-TET	1			
AMP-AUG-CHL-CIP-GEN-KAN-NAL-SMX-SXT			1	
AMP-AUG-CHL-CRO-TET-KAN-SMX-STR-SXT		2		
AMP-AUG-CHL-CIP-KAN-NAL-SMX-SXT			1	
AMP-CHL-GEN-KAN-NAL-SMX-SXT-TET			1	
AMP-AUG-CHL-KAN-SMX-STR-SXT-TET			1	1
AMP-AUG-CHL-CIP-SMX-STR-SXT-TET	1			
AMP-AUG-CHL-GEN-KAN-SMX-SXT-TET	1			
AMP-CHL-GEN-KAN-NAL-SMX-SXT-TET		2		
AMP-AUG-KAN-SMX-STR-SXT-TET	1	1		
CHL-NAL-KAN-SMX-STR-SXT-TET				1
CHL-KAN-SMX-STR-SXT-TET		1		
AMP-AUG-KAN-SMX-SXT-TET	2			
CHL-KAN-SMX-STR-SXT-TET	1			
CHL-NAL-SMX-STR-SXT	1			
CHL-SMX-SXT				1

AMP, ampicillin; AUG, amoxicillin–clavulanic acid; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; SMX, sulfamethoxazole; STR, streptomycin; SXT, trimethoprim–sulfamethoxazole; TET, tetracycline.